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# Klotho, a New Marker for Osteoporosis and Muscle Strength in $\beta$ -Thalassemia Major

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## Riassunto

Le  $\beta$ -talassemie sono un gruppo di malattie del sangue ereditarie caratterizzate da una ridotta o assente sintesi di  $\beta$ -globina, con fenotipi variabili che vanno da una grave anemia ad individui clinicamente asintomatici. Il ritardo di crescita si osserva frequentemente e fratture da fragilità sono comuni in questi pazienti. L'osteoporosi colpisce circa il 51% dei pazienti e circa il 45% l'osteopenia.

Negli ultimi anni, i ricercatori hanno focalizzato l'attenzione su un gene chiamato  $\alpha$ -Klotho (Klotho). Il gene Klotho codifica per una proteina che si esprime in reni, ghiandole paratiroidi e plesso coroidale. Topi knock-out sviluppavano un fenotipo da 'invecchiamento' che comprendeva, tra le altre condizioni, riduzione della densità ossea, ritardo di crescita e ipogonadismo. La proteina è presente nel sangue, urine e nel liquido cerebrospinale ed è coinvolta nella omeostasi del calcio nel rene.

Ci sono pochi studi clinici che collegano i livelli di Klotho secreto nel sangue periferico e soggetti umani. Nessuno in pazienti con  $\beta$ -talassemia. Lo scopo di questo studio è stato quello di analizzare le possibili correlazioni tra livelli plasmatici della proteina Klotho e l'osteoporosi, scarsa forza muscolare e fratture, nei pazienti con  $\beta$ -talassemia major.

Un totale di 106 pazienti con  $\beta$ -talassemia major e 95 donatori di sangue sani sono stati arruolati. I pazienti con  $\beta$ -talassemia major avevano un minor livello di Klotho rispetto ai controlli sani. Klotho era più basso nei pazienti con osteopenia / osteoporosi. Inoltre, un basso livello di proteina aumenta la probabilità di frattura da fragilità. La forza muscolare è direttamente correlata I livelli di Klotho (fino a 580 pg/ml).

Questa analisi suggerisce che età o addirittura malattie legate all mancanza di Klotho, possono giocare un ruolo chiave nella catena di eventi che portano a sarcopenia e osteoporosi, componenti della sindrome da dismobilità. Inoltre, questo studio identifica Klotho come un potenziale fattore di

rischio per sindromi da dismobilità e la sua complicazione clinicamente rilevante, cioè fratture da fragilità.

## Abstract

$\beta$ -thalassemia syndromes are a group of hereditary blood disorders characterized by reduced or absent  $\beta$ -globin synthesis, resulting in variable phenotypes ranging from severe anemia to clinically asymptomatic individuals. Growth retardation is observed frequently and fragility fractures are common in these patients. Osteoporosis affects approximately 51% of the patients with another 45% suffering from osteopenia.

In recent years, researchers have focused the attention on a gene called  $\alpha$ -Klotho (Klotho). The Klotho gene encodes a protein that is expressed in kidney, parathyroid gland and choroid plexus.

Klotho protein is present in blood, urine and cerebrospinal fluid and it is involved in calcium homeostasis in the kidney. Re-absorption of calcium in the distal tubule of the kidney. knock-out mutants for the gene. These animals developed an 'aging' phenotype which included, among other conditions, reduced bone density, growth retardation, hypogonadism

There are very few clinical studies that link the levels of secreted Klotho in peripheral blood and phenotype in humans and none of them in  $\beta$ -thalassemia subjects.

Aim of this study was to analyze possible correlations between plasma level of Klotho protein and osteoporosis, poor muscle strength and fractures in patients with  $\beta$ -thalassemia major.

A total of 106  $\beta$ -thalassemia major patients and 95 healthy blood donors were enrolled. Patients with  $\beta$ -thalassemia major had lower level of Klotho than healthy controls. Klotho was lower in patients with osteopenia/osteoporosis.

Moreover, a low level of protein increased the probability of fragility fracture. The muscle strength was found directly correlated to Klotho (up to 580 pg/ml).

This analysis suggests that age- or even a disease-related decrease of Klotho may play a key role in the chain of events producing sarcopenia and osteoporosis, components of the dysmobility syndrome. Furthermore, this study identifies Klotho as a potential risk factor for dysmobility

syndrome and its clinically relevant complication, namely fragility fractures.

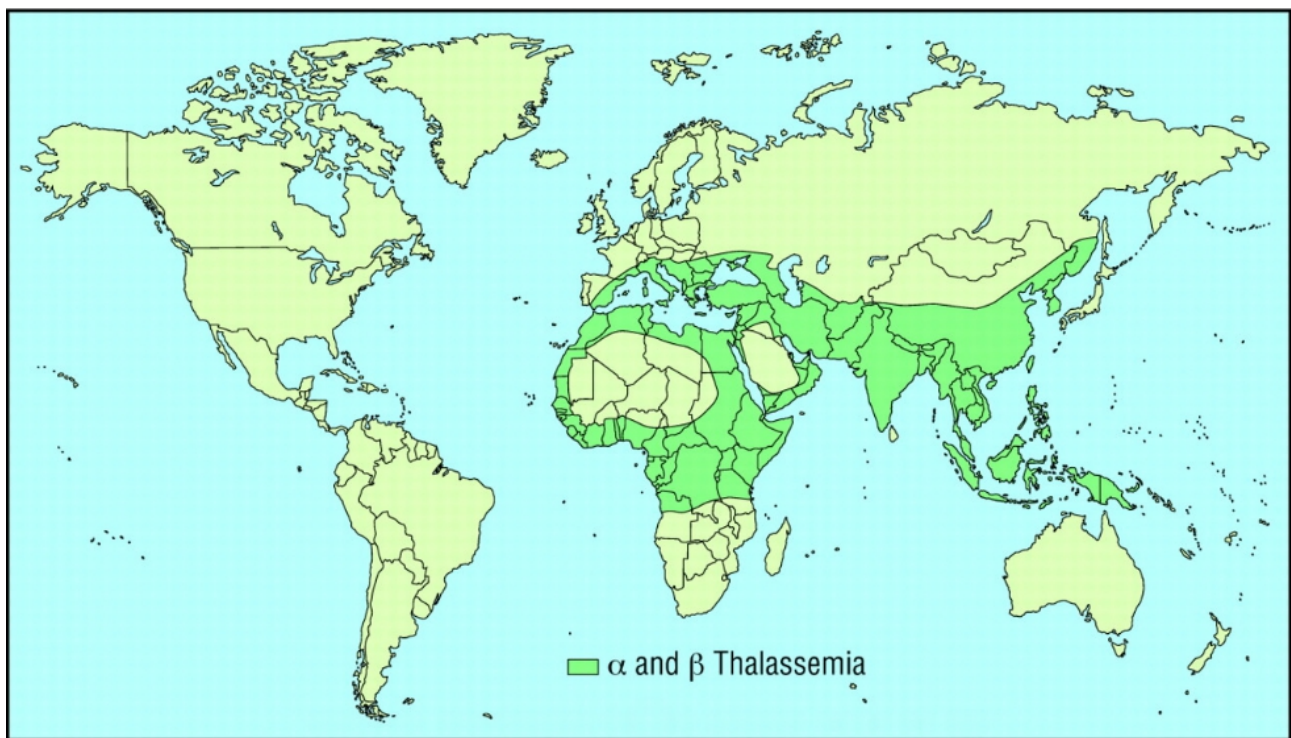
# 1. Introduction

## *1.1 Thalassemias*

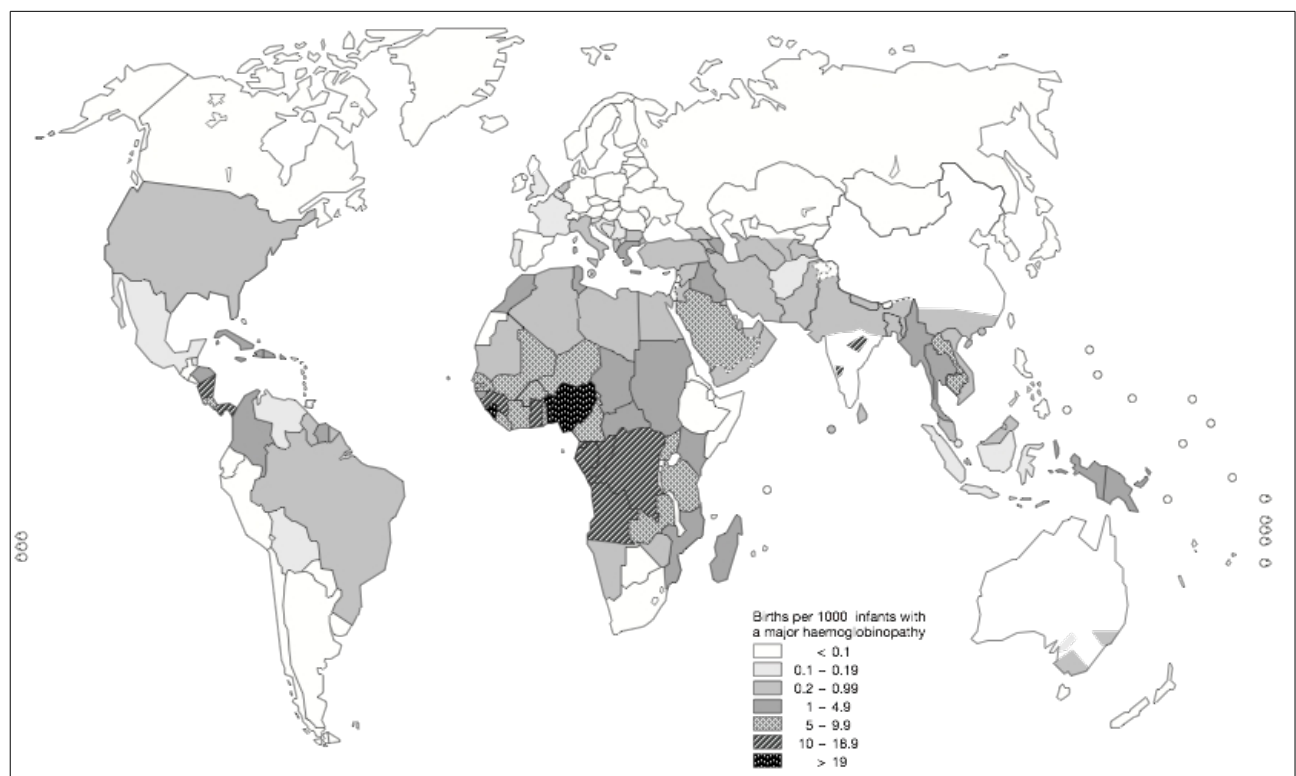
Thalassemias are a group of congenital anemia that has an impaired synthesis of one or more of the globin subunits of the normal human hemoglobins (Hbs). The primary defect is usually quantitative, consisting of the reduced or absent synthesis of normal globin chains. The consequence is a hemoglobin with imbalanced globin chains.<sup>(1)</sup> There are different types of thalassemia and that depends on the chains involved: alpha ( $\alpha$ ), beta ( $\beta$ ), gamma ( $\gamma$ ), delta ( $\delta$ ), delta-beta ( $\delta\beta$ ), or epsilon-gamma-delta-beta ( $\epsilon\gamma\delta\beta$ ). Advances in DNA analysis has permitted to understand the gene structure, function and the characterization of the molecular basis that lead to deficient globin synthesis. The thalassemias are the consequence of a large number of different molecular defects leading to a variety of clinical and hematological phenotypes.

Thalassemia is considered the most common genetic disorder worldwide.<sup>(2)</sup> It spreads from the Mediterranean basin through the Middle East, Indian subcontinent, Burma, Southeast Asia, Melanesia, and the islands of the Pacific (Figure 1). Based on the Hereditary Disease Program of the World Health Organization (WHO) and on local surveys the estimated number of subjects with or carrying hemoglobin disorders in the world is about 269 million (Figure 2).





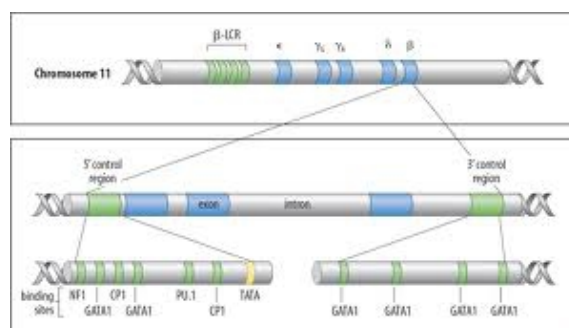
**Figure 1. Distribution of  $\alpha$  and  $\beta$  Thalassemia worldwide.** They spread from the Mediterranean basin through the Middle East, Indian subcontinent, Burma, Southeast Asia, Melanesia, and the islands of the Pacific.



**Figure 2. Global distribution of haemoglobin disorders, in terms of births of affected infants per 1000 births.**

## 1.2 $\beta$ -Thalassemia

The  $\beta$ -thalassemia genes are present in about 3% of the world's population (150 million people). In Europe they are particularly prevalent in Italy and Greece. In Italy, the highest prevalence of carriers was found in Sardinia (10.3%), the delta region of the Po River near Ferrara (8%), and Sicily (5.9% with an almost equal distribution over the entire island) <sup>(3-5)</sup> In Greece, the prevalence varies considerably, ranging from <5% to nearly 15% in the southern and central areas.<sup>(6,7)</sup> In Cyprus, one individual in seven is a carrier of  $\beta$ -thalassemia and 1 individual in 1000 is currently homozygous. In Sardinia the incidence of homozygous  $\beta$ -thalassemia is 1 in 250 live births. <sup>(8)</sup> The  $\beta$ -globin gene is located in the short arm of chromosome 11 in a region containing also the  $\delta$  gene, the embryonic  $\epsilon$  gene, the fetal G gamma and A gamma genes, and the pseudogene  $\beta 1$  (Figure 3). <sup>(9)</sup> The five functional globin genes are arranged in the order of their developmental expression. The  $\beta$ -globin genes are subject to a very complex regulatory mechanism, acting at the level of single genes as well as of the entire  $\beta$  cluster.

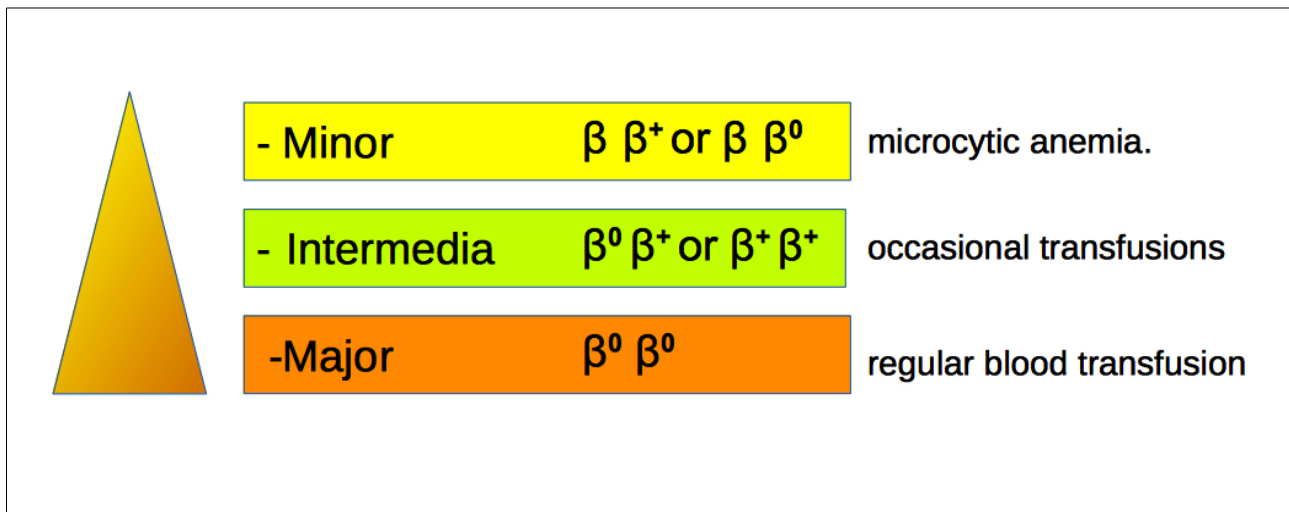


**Figure 3. The globin gene.** The five functional globin genes are arranged in the order of their developmental expression.

$\beta$ -Thalassemia mutations result in either a complete absence of  $\beta$ -globin chains ( $\beta^0$ -thalassemia) or in a largely variable reduction of  $\beta$ -globin synthesis ( $\beta^+$ -thalassemia). More than 200 different mutations producing  $\beta$ -thalassemia have been so far described; the large majority are point mutations in functionally important sequences of the  $\beta$ -globin gene, while in contrast to  $\alpha$ -

thalassemia, gene deletion is a rare cause of  $\beta$ -thalassemia.

As a consequence of  $\beta$ -chain impairment, there is an excess of unbound  $\alpha$  globin chains in erythroid precursors that precipitate in the bone marrow, leading to their premature death and to ineffective erythropoiesis.  $\beta$ -thalassemia phenotypes are variable, ranging from the severe transfusion dependent thalassemia major to the mild form of thalassemia intermedia (Figure 4).<sup>(10)</sup> Thalassemia intermedia does not require transfusion or only sporadic or intermittent transfusions. Thalassemia minor indicates the heterozygous state, which is usually completely asymptomatic. Thalassemia minima was used in the Italian literature to indicate a carrier in whom no hematologic symptoms. Sometimes the term of thalassemia minima was used to indicate the condition of silent carrier.



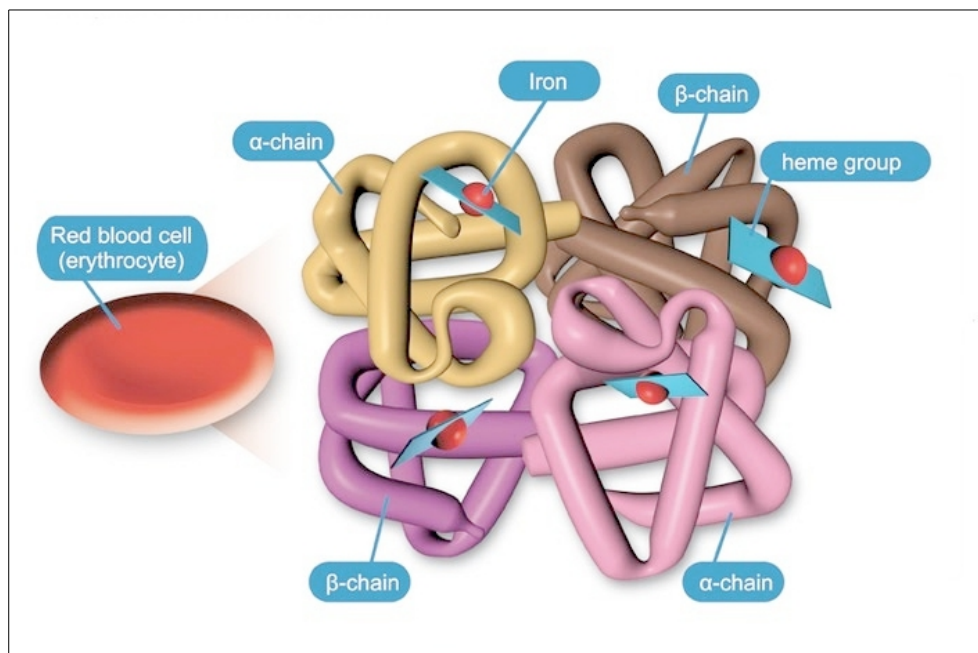
**Figure 4. Correlation genotype-phenotype in  $\beta$ -Thalassemia.** *Thalassemia minor* indicates the heterozygous state, which is usually completely asymptomatic. *Thalassemia intermedia* is the term used to designate a form of anemia that usually does not require transfusion or only sporadic or intermittent transfusions. The most severe form is *Thalassemia major* and is characterized by transfusion-dependent anemia.  $\beta^0$  indicates no synthesis of the  $\beta$  chain.  $\beta^+$  indicates reduced synthesis and  $\beta$  the wild-type.

### 1.2.1 $\beta$ -Thalassemia Major

The  $\beta$ -thalassemia syndromes represent the most relevant forms of thalassemia and the different forms are based on clinical severity. The most severe form is defined  $\beta$ -thalassemia major and it is a transfusion-dependent anemia. There are about 3,500 individuals with thalassemia major in Greece and 4300 in Italy. <sup>(11)</sup>

Originally, there was a disease called *anemia splenica infantum* that included several conditions, often not well distinguished from one another. These children were usually born normally and grew normally until the second half of the first year, when they were noticed to become paler and paler, and to develop an enormous abdomen, containing a spleen that could extend from a few centimeters below the left costal margin to the iliac crest and below, sometimes visible from the outside. Bone deformities, especially of the skull, soon appeared, giving the children a distinctive “mongolian” appearance. <sup>(12,13)</sup> The disease was often present in more than one sibling, or, more frequently, other siblings had previously died of the same disease. The first systematic descriptions of what was going to be identified as thalassemia major came from Cooley and Lee <sup>(12)</sup> from Michigan, who observed the disease in Italian and Greek children, and from Maccanti, a pediatrician from Ferrara, Italy, who also noted that the children were often coming from malarial areas near the Po river. <sup>(12,14)</sup> Anemia, leukocytosis, and normo-blastemia were always present. Both groups tried unsuccessfully the entire armamentarium of therapies then available (arsenium, fresh veal bone marrow, sunshine, the quartz lamp, cod liver oil, and, of course, iron) and even blood transfusions, which were helpful but short lasting in one patient but caused increased hemolysis in another. <sup>(14)</sup> This is not surprising, considering the very limited blood matching available at the time and the already enormous size of the spleen at presentation. Splenectomy and roentgen irradiation of the spleen were also performed without benefit. All the children died shortly after presentation. Detailed autopsic data, showing peculiar abnormalities in the bones and spleen fibrosis, are available. Almost at the same time, Rietti, also from Ferrara, had reported three adult patients, two of whom were father and son, who

presented with “primitive hemolytic jaundice” associated with decreased osmotic fragility. <sup>(15)</sup> Anemia, microcytosis, anisocytosis, and basophilic stippling were noted. In 1932, in consideration of the Mediterranean origin of the patients affected by Cooley anemia, Whipple and Bradford proposed the name of *thalassemia*, from the Greek word *thalassa*, meaning sea. <sup>(16)</sup> Subsequently, the severe and the mild form of thalassemia were denominated *thalassemia major* and *minor*, respectively. Unfortunately, the lack of communication between the two sides of the Atlantic made the research in this field, as in others, proceed slowly in parallel. <sup>(17,18)</sup> In 1940, Wintrobe reported the presence of a familial hemopoietic disorder in adolescents and adults of Italian origin, while in Italy, between 1943 and 1947, Silvestroni and Bianco defined the hematologic, clinical, and epidemiologic characteristics of thalassemia minor and its relationship with thalassemia major. <sup>(19)</sup> A detailed report of that research can be found in a recent, comprehensive book on thalassemia by Ida Bianco Silvestroni. <sup>(20)</sup> The picture was further clarified by the identification of HbA<sub>2</sub> and its increase in the parents of patients affected by thalassemia major. <sup>(21)</sup> Between 1956 and 1961 the chemical structure of hemoglobin was defined, (Figure 5) and soon the complete sequences of globin chains were clarified. <sup>(22)</sup>



**Figure 5. Structure of hemoglobin.** Each erythrocyte (RCB) contains about 270 million hemoglobin molecules.

The idea of thalassemia resulting from a defect in the production of adult hemoglobin is due to the contribution of several authors. Globin chain synthesis was able to confirm this hypothesis. <sup>(23)</sup>

For many years after the description of thalassemia major as a clinical entity, the main therapy was limited to blood transfusion when symptoms of anemia were so severe as to incapacitate the patient. The corresponding levels of hemoglobin (Hb) were different in different patients, but varied between 6 and 7 g/dl. As a consequence of continuous anemia, erythropoiesis, although inefficient, was intense, the bone marrow underwent an enormous expansion, and the plasma volume increased greatly. Also liver and spleen increased in size as a consequence of both extramedullary erythropoiesis and hemolytic activity in the reticuloendothelial tissue. The bone deformities caused by the expanded marrow are typical of thalassemia and gave to all the poorly transfused patients similar features.

#### ***1.2.1.1 Clinical Complications***

For patients with  $\beta$ -Thalassemia major, the clinical complications are related to the degree of imbalance between the  $\alpha$  and non- $\alpha$  globin chains. When not properly treated, several complications can arise and jeopardize the health of the patient. These complications include: growth retardation, poor musculature, leg ulcers and peculiar bone deformities due to the bone marrow expansion. <sup>(1)</sup>

Cardiac problems and liver disease are also frequent as a consequence of iron overload.

Complications of the disease include heart problems (heart failure and arrhythmia), liver disease, due to transfusional iron overload and transfusion transmitted viral infections, endocrine problems including hypogonadism in over 50% of the patients, diabetes and hypothyroidism (Figure 6). Growth retardation is observed frequently and fragility fractures are common in these patients. The reduced bone mineral density and consequent susceptibility to fractures has been attributed, in addition to hyperactivity of the bone marrow, to endocrine dysfunction, iron overload, chelation therapy, vitamin D deficiency and lack of exercise.

#### ***1.2.1.1.1 Cardiac and Liver Problems***

Heart failure and arrhythmias are responsible for over 70% of the deaths. <sup>(45)</sup> Cardiac complications can range from involving both ventricles to pulmonary hypertension. The development of T2\* cardiac magnetic resonance technique allows the detection of iron in the heart and liver. It helps to identify patients at high risk of heart failure and arrhythmia from myocardial siderosis. <sup>(25)</sup>

The liver is the main site of iron deposition. Moreover, patients can be at higher risk of hepatotropic virus infections. <sup>(26)</sup> Liver iron overload can lead to fibrosis and cirrhosis. Fibrosis correlates directly with age, number of unit of blood transfused and liver iron concentration. <sup>(27)</sup>

#### ***1.2.1.1.2 Anemia***

The early symptoms of the disease appear usually in the first year of life, at the time when the synthesis of  $\gamma$  chains is not replaced by the synthesis of  $\beta$  chains. In an ethnically composite population of transfusion-dependent children diagnosed in the United Kingdom, the mean age at presentation was reported to be 6 months, while in a study from Greece the age was 13.1 months, ranging from 2 to 36 months. <sup>(28)</sup> In Sardinia the disease was recognized at around 8 months in patients with transfusion-dependent thalassemia but at age 2 years in non-transfusion-dependent children. <sup>(29)</sup> The age at diagnosis is influenced by the molecular defect and by the degree of suspicion of the treating physician. Pallor is usually the first sign, accompanied by splenomegaly of various severity, fever, and failure to thrive.

#### ***1.2.1.1.3 Bone Deformities***

Un-transfused or poorly transfused patients with thalassemia develop typical bone abnormalities that were described already in the first reports of the disease and that are due to the extremely increased erythropoiesis, with consequent expansion of the bone marrow to 15-30 times normal.

The skull is large and deformed by frontal and posterior bossing with the diploe increased in thickness to several times normal. The outer and inner tables are thin and the trabeculae are arranged in vertical striations, resulting in a “hair on end” appearance. A peculiar, stratified appearance of the skull has been reported.<sup>(30)</sup> The zygomatic bones are prominent, the base of the nose is depressed and pneumatization of the sinuses is delayed. Overgrowth of the maxilla produces severe malocclusion, with a rodent like appearance. Metatarsal and metacarpal bones are the first to expand as a consequence of increased erythropoiesis. The ribs are broad, often with a “rib-within-rib” appearance, and the vertebral bodies are square. The trabeculation of the medullary space gives the bones a mosaic pattern. Shortening of long bones is frequent, as a result of premature fusion of the humeral and femoral epiphyseal lines.<sup>(31,32)</sup> Extramedullary erythropoiesis gives rise to masses that protrude from bones where red marrow persists.<sup>(33)</sup>

Overgrowth from the vertebral bodies has been reported to cause cord compression and paraparesis.<sup>(34)</sup> (320) Ear impairment due to extramedullary marrow growing in the middle ear and progressive visual loss caused by compressive optic neuropathy have been reported.<sup>(35,36)</sup> This kind of picture is more often present in patients with thalassemia intermedia, in whom transfusions are avoided at the price of intense autologous marrow hyperactivity. Improvement in the radiological bone appearance in the cohorts of patients who have, since an early age, undergone regular transfusions has been striking. The lack of severe skull deformities is reflected in the mildness of thalassemic features that are now observed in most patients.

#### ***1.2.1.1.4 Osteoporosis***

Susceptibility to fractures is very common as a result of reduced bone mineral density.<sup>(37)</sup> Mineral density is usually investigated with Dual-energy X-ray Absorptiometry (DXA) at the spinal (L1 to L4), total hip and femoral neck level. Osteoporosis is defined as a decrease in bone mineral density  $\geq 2.5$  SD below the young adult mean value, while a decrease between  $-1$  and  $-2.5$  SD is defined as



osteopenia.<sup>(38)</sup> Osteoporosis in thalassemia affects about 51% of the patients with an additional 45% affected by osteopenia.<sup>(39)</sup> The pathogenesis of osteoporosis in thalassemia major is multifactorial and results from a variety of genetic and acquired factors.

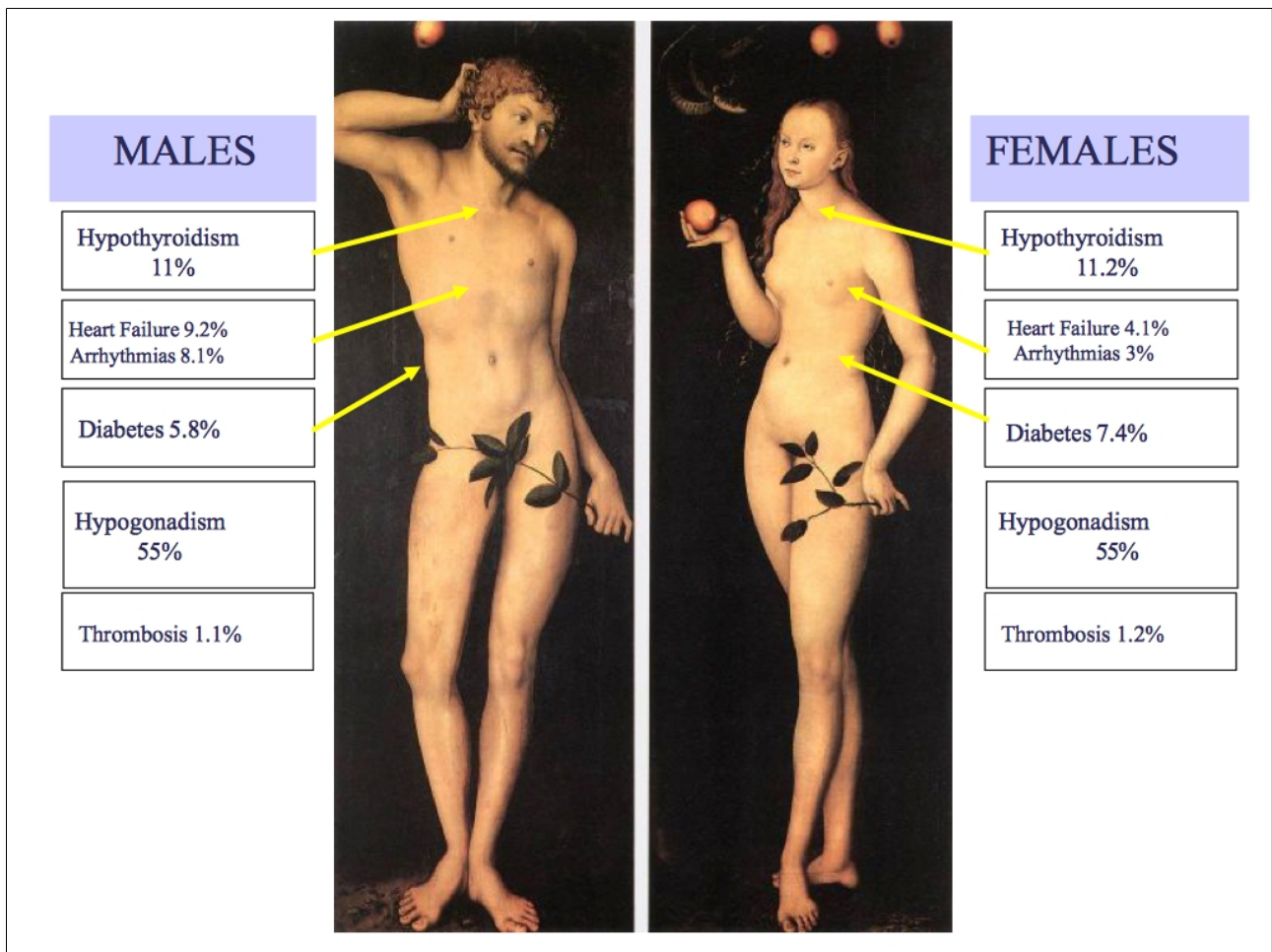
Vitamin D receptor (VDR) polymorphisms were found to be a risk factor for bone mineral damage, low bone mineral density and short stature in prepubertal and pubertal patients.<sup>(40)</sup> Nevertheless, different studies on genetic polymorphisms have given contradictory results.<sup>(41,42)</sup> Acquired factors include the primary disease itself causing ineffective hematopoiesis with progressive bone marrow expansion, and several secondary factors such as endocrine dysfunction, iron overload and chelation therapy, vitamin deficiencies and decreased physical activity.<sup>(43,44)</sup> In particular, vitamin D deficiency is frequent among adolescents.<sup>(45)</sup> The reduced bone mineral density and consequent susceptibility to fractures has been attributed, in addition to hyperactivity of the bone marrow, to endocrine dysfunction, iron overload, chelation therapy, vitamin D deficiency and lack of exercise.<sup>(46)</sup> Conventional therapy for osteoporosis in thalassemia includes Vitamin D and Calcium supplements. However, supplementation with calcium and vitamin D has not been demonstrated to reduce the risk of fragility fractures while it has been shown to increase the risk of kidney stones.<sup>(47)</sup> Reduced and irregular mineralization of the bone has been found using DXA in thalassemia patients with and without clinically evident bone abnormalities.<sup>(48)</sup> Osteoporosis is a progressive disease, thus early detection, prevention, and treatment are essential for effective control of this potentially debilitating condition.<sup>(43)</sup> In thalassemia patients, the reduced osteoblastic activity is accompanied by a comparable or even greater increase in bone resorption. An effective treatment is given by bisphosphonates that are inhibitors of osteoclast activation.<sup>(49)</sup> However, data about their anti-fracture efficacy is still missing.<sup>(50)</sup>

#### ***1.2.1.1.5 Growth Retardation***

As a consequence of the frequent transfusions, the patients are subject to iron overload. Iron deposition is the main cause of damage to the endocrine glands, directly or through the hypothalamic-pituitary axis. Direct damage to almost all endocrine glands has been demonstrated histologically and by MRI. High ferritin levels, poor compliance with chelation, and splenectomy increase the risk of being affected by all endocrinopathies. <sup>(51–53)</sup>

Stunted growth is common in thalassemia and it is typically characterized by normal growth during childhood, decreased growth velocity at the end of the first decade of life and evident growth failure at the expected age of puberty in those patients who lack sexual maturation. Poor pubertal growth, however, was found to be present also in a group of thalassemic patients regardless of hypogonadism. <sup>(54)</sup>

Sitting height is reduced as a consequence of spinal growth abnormalities. A recent survey from the US found that approximately 25% of patients with a thalassemia syndrome, <sup>(45)</sup> had short stature. In children, however, growth was mildly affected and final height was close to midparental height. A report from India, where the majority of adult patients reached a height below 150 cm, highlights the importance of adequate hemoglobin levels and regular transfusion since childhood for normal growth, <sup>(55)</sup> confirming observations made in the USA and Europe before the introduction of hypertransfusion. <sup>(56)</sup>

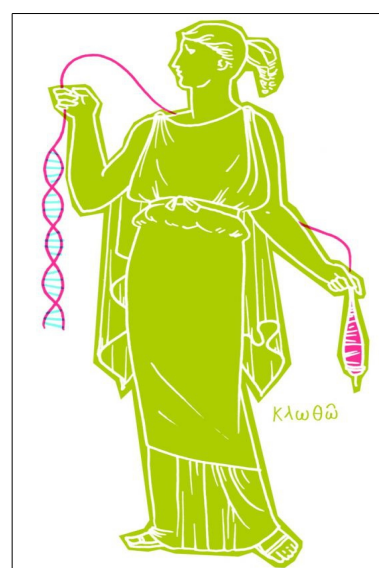


**Figure 6. Clinical complications in patients with  $\beta$ -Thalassemia major.** The clinical complications are related to the degree of imbalance between the  $\alpha$  and non- $\alpha$  globin chains. When not properly treated, several complications can arise and jeopardize the health of the patient.

### 1.3 Klotho

In recent years, researchers have focused the attention on a gene called  $\alpha$ -Klotho (referred as Klotho throughout the dissertation).<sup>(57)</sup> The Klotho gene encodes a protein that is expressed in kidney, parathyroid gland and choroid plexus. The name comes from one of the Fates, who according to mythology was responsible for cutting the thread of life (Figure 7).

The Klotho gene was originally identified as a gene mutated in the Klotho mouse. The Klotho mouse displays complex phenotypes resembling human premature aging syndromes including shortened life span, poor growth, hypogonadism, skin atrophy, muscle atrophy, premature thymic involution, osteopenia, pulmonary emphysema, vascular calcification, and soft tissue calcification among others, in an autosomal recessive manner (Figure 8).<sup>(58)</sup> The Klotho gene was isolated by positional cloning and was found to encode a novel single-pass transmembrane protein of about 1012 aminoacids (aa) that is expressed predominantly in the kidney.<sup>(59)</sup> Expression of the Klotho gene in the Klotho mouse was undetectable by northern blot analysis, indicating that Klotho deficiency is responsible for the syndrome resembling aging. By contrast, transgenic mice that overexpress Klotho exhibited increased resistance to oxidative stress<sup>(60)</sup> and significant extension of life span.<sup>(61)</sup> These observations suggest that the Klotho gene might function as an aging suppressor gene.



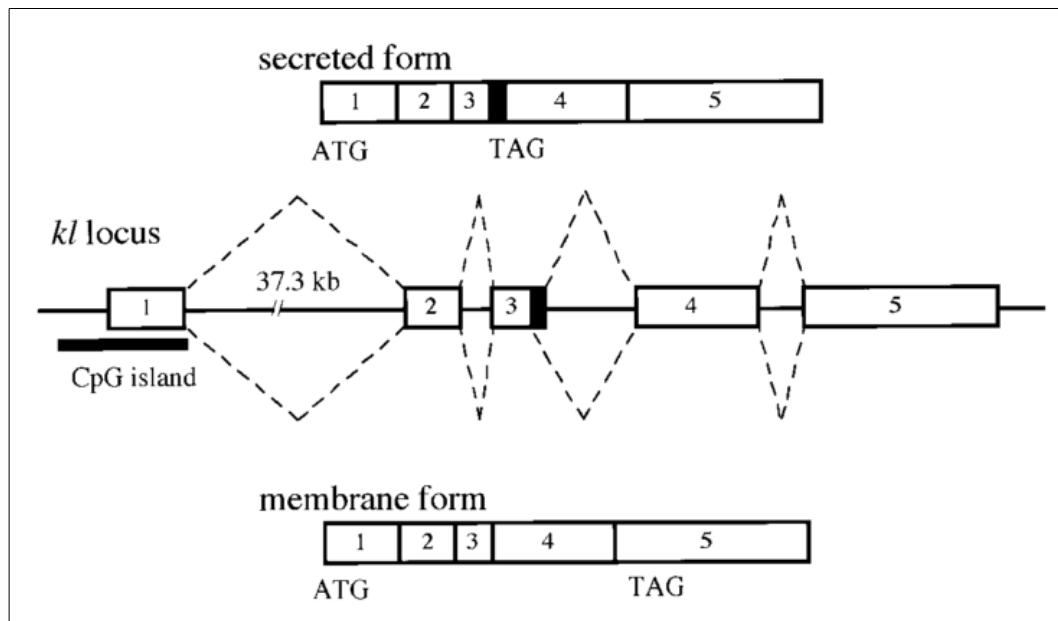
**Figure 7. Klotho in Greek mythology.** She is the youngest of the Three Fates including her sisters Lachesis and Atropos. Klotho was responsible for spinning the thread of human life. This power enabled her not only to choose who was born, but also to decide when gods or mortals were to be saved or put to death.



**Figure 8. Comparison of 8-week old Klotho-knock-out mice (kl/kl) to the wild type.** kl/kl mice with original (agouti) and with Balb/c background (albino) are shown with their +/+ littermates. Up to 3 to 4 weeks of age, kl/kl mice grow normally and are indistinguishable from their +/+ or kl/+ littermates. At that time, kl/kl mice begin to show growth retardation and die prematurely at 8–9 weeks of age. <sup>(58)</sup>

### ***1.3.1 The Gene***

The mouse Klotho gene (OMIM: 684824) is composed of 5 exons and 4 introns and resides on chromosome 13q13.1 with a size of over 50 kb. <sup>(58)</sup> The transcript of the mouse Klotho gene is about 5.2 kb. The size of the human and rat Klotho gene transcript is similar to that of the mouse (5.2 kb). <sup>(62–64)</sup> Its promoter region lacks a TATA-box and contains four potential binding sites for Sp1 (a human transcription factor involved in gene expression in the early development of an organism). <sup>(62)</sup> In the third exon, there is an alternative splicing at an internal splice donor site (Figure 9).



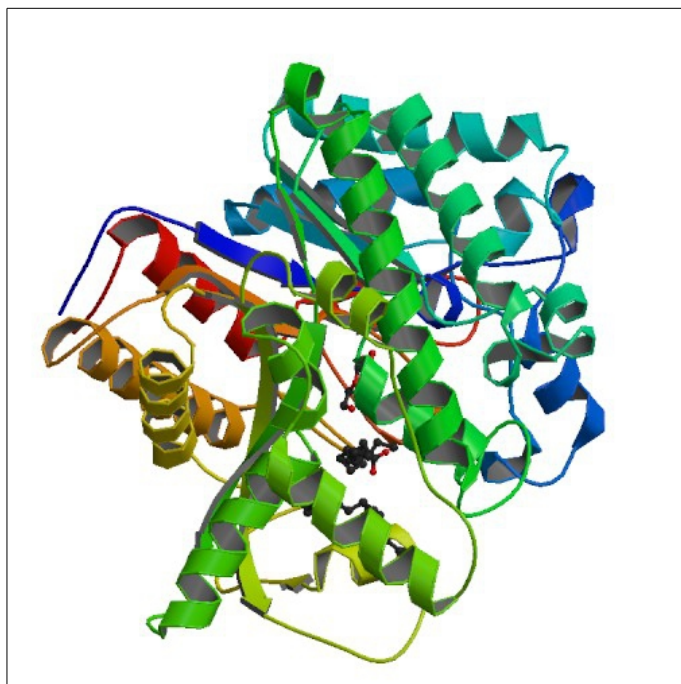
**Figure 9. Schematic of the genomic organization of the human Klotho (kl) gene.** Two different transcripts encoding the secreted and membrane forms of the kl protein are generated through alternative RNA splicing at exon 3. It is located on chromosome 13q12 with a size of over 50 kb. The white boxes indicate the exons. The black box beside exon 3 indicates the site of splicing. It is still not clear if the secreted form generated by the alternative splicing carries both KL domains or only one.

Two transcripts arise from this alternative RNA splicing: a transmembrane and another secreted form of Klotho protein. This alternative splicing secreted form seems to have only one of the two domains. Moreover, it represents a little percentage of the total circulating protein. Klotho expression is influenced by many physiological and pathological conditions. For example, Klotho expression is minimal in prenatal life, but markedly augmented after birth in the rat kidney.<sup>(64)</sup> With aging, Klotho expression decreases in the heart sinoatrial node and the liver.<sup>(63,65)</sup> In the white matter of the rhesus monkey, Klotho protein expression is also significantly decreased with age.<sup>(66)</sup> By using two microsatellite markers flanking the Klotho gene and DNA sequencing, Arking *et al.* demonstrated that a functional variant of Klotho (KL-VS) was associated with human survival and longevity (defined as postnatal life expectancy greater than 75 years).<sup>(67)</sup> Klotho mRNA expression is also significantly decreased in some disease conditions. It is speculated that Klotho

has high affinity to yet unidentified receptors. The extracellular domain of Klotho may bind to its putative receptors that express on the cell surface, and this binding may activate a signaling pathway that cross-talks with the insulin/IGF1 signaling pathway, but the precise mechanism remains to be determined. However, Klotho protein regulates insulin/IGF1 could involve resistance to oxidative stress at the cellular and organismal levels in mammals. <sup>(68)</sup> Because mammalian fork-head box O (FOXO) transcription factors, FOXO1, FOXO3a, and FOXO4, are negatively regulated by insulin/IGF-1 signaling, activation of insulin/IGF-1 signaling leads to phosphorylation and activation of a serine-threonine kinase Akt, which in turn phosphorylates FOXOs. <sup>(69)</sup> Phosphorylated FOXOs are excluded from the nucleus and inactivated.

### ***1.3.2 The Protein***

The single-pass transmembrane protein has an extracellular domain of about 980 aa. About 21 aa form the membrane spanning domain and about 11 aa the intracellular carboxyl terminus (Figure 10). Klotho exists also as a secreted form that is found to predominate over the membrane form in kidneys. It concludes that the major Klotho gene product is not the membrane protein but the secreted form from the enzymatic cleavage. <sup>(62)</sup> Klotho gene encodes a type 1 membrane glycoprotein of about 130 KDa. Expression was seen in renal tubul cells, parathyroid secretory cells and epithelial cells of the choroid plexus. The extracellular domain is made of two internal repeats KL1 and KL2, that display similarity to the family 1  $\beta$  -glycosidases. <sup>(70)</sup> Glycosidases are the enzymes that hydrolyze the glycosidic bond between two carbohydrates or between carbohydrate and non-carbohydrate moieties. <sup>(71)</sup>

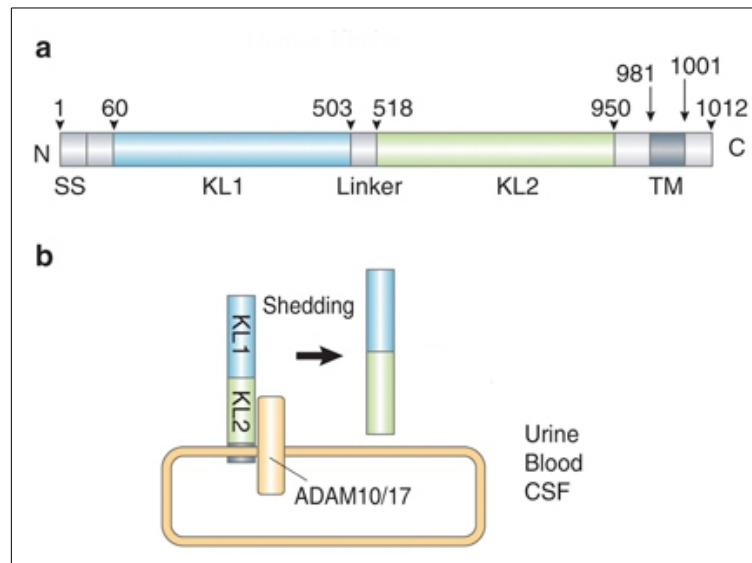


**Figure 10. 3D view of the Klotho protein.** The extracellular domain is made of two repeats KL1 and KL2, that display similarity to the family 1  $\beta$ -glycosidases. (www.rcsb.org)

Family 1 glycosidases operate with a molecular mechanism leading to overall retention of the anomeric configuration and involving the formation and breakdown of a covalent glycosyl enzyme intermediate. Two Glu residues are directly involved in this catalytic mechanism, one acting as a nucleophile and the other as an acid/base.<sup>(72)</sup> However, Klotho does not possess these two catalytic glutamic acids. In the KL1 region, the nucleophilic Glu residue is present, but the acid/base Glu residue is replaced by an Asn residue. Inversely, in the KL2 region, the acid/base Glu residue is present, but the nucleophile is replaced by Ala and Ser residues in mouse and human Klotho, respectively.<sup>(58)</sup> The enzymatic activity of Klotho showed maximal activity at pH 5.5, a value close to the pH 6.0 optimal for bovine liver  $\beta$ -glucuronidase. Although the enzymatic activity of the  $\beta$ -glucuronidase was inhibited by citrate buffer, Klotho was unaffected. In addition, the enzymatic activity of Klotho was reduced by known inhibitors of  $\beta$ -glucuronidases. Tohyama *et al.*



concluded that Klotho behaved like a  $\beta$ -glucuronidase having the ability to catalyze the breakdown of complex carbohydrates.<sup>(73)</sup> The membrane Klotho undergoes a proteolytic cleavage on the cell surface by membrane-anchored A Disintegrin and Metalloproteinases (ADAM)-17 and -10 (Figure 11).<sup>(74)</sup> The protein is shed into the urine, blood, and cerebrospinal fluid. This secreted Klotho elicits biological effects on target cells suggesting that it functions as a circulating hormone.<sup>(74,75)</sup>



**Figure 11. Domain structure and topology of Klotho.** (a) Domain structure of human Klotho. Not drawn to scale. SS, KL1, KL2, TM indicate signal sequence, first and second Klotho repeat, and transmembrane domain, respectively. (b) Membrane topology of Klotho. The entire extracellular domain consisting of KL1 and KL2 repeats is cleaved by ADAM10 or ADAM17 and released into urine, blood, or cerebrospinal fluid (CSF).

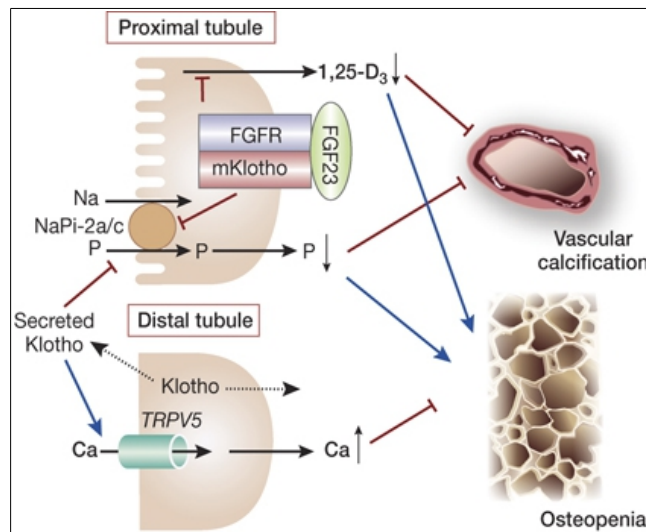
## 1.4 Klotho, Phosphate and Vitamin D Regulation

Recent studies have revealed multiple functions of Klotho protein. Most notably, Klotho protein forms a complex with several FGFR isoforms significantly enhances their affinity for FGF23.<sup>(76)</sup> This discovery was prompted by the fact that FGF23-deficient mouse and Klotho-deficient mouse displayed almost identical phenotypes. They showed multiple aging-like phenotypes and

metabolic abnormalities characterized by low blood glucose, high blood phosphate, and high active vitamin D levels in the blood. <sup>(77,78)</sup> These findings suggested that Klotho and FGF23 worked in a common molecular pathway. FGF23 required Klotho as a co-receptor for its biological activity that induced renal phosphate excretion and suppression of the active vitamin D (1,25-dihydroxy-vitamin D3) biosynthesis, which promotes absorption of calcium and phosphate from intestine. Thus, activation of the FGF23- Klotho axis induced negative phosphate balance not only by increasing renal phosphate excretion but also by decreasing intestinal phosphate absorption. <sup>(79)</sup>

### ***1.5 Klotho and Calcium Regulation***

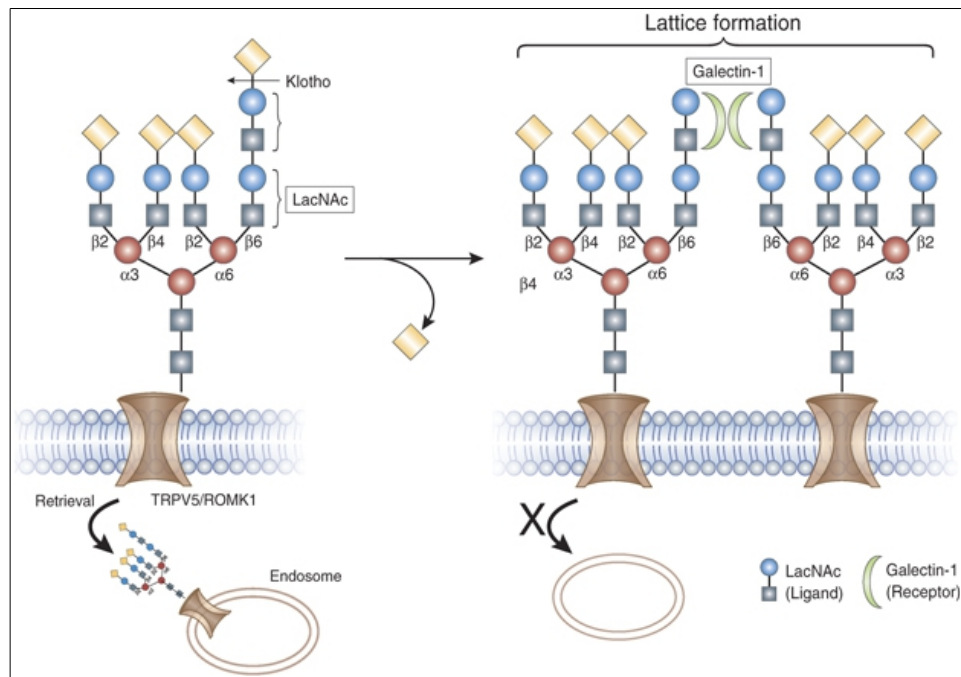
Calcium is absorbed from the diet to enter the blood compartment and excreted through the urine, which is tightly controlled by the kidney through the reabsorption of Calcium from the filtrate (Figure 12). The majority of Calcium reabsorption takes place in the proximal tubules and the thick ascending limb of Henle through the passive paracellular route. <sup>(80)</sup> Transepithelial Calcium reabsorption occurs in a series of consecutive steps: driven by a highly favorable electrochemical driving force Calcium enters the cell through the apical transient receptor potential vanilloid 5 (TRPV5) Calcium channel. <sup>(81)</sup> TRPV5 belongs to the TRP superfamily, which consists of cation-selective ion channels with similar molecular structures. <sup>(82)</sup> Of all TRP channels, TRPV6 holds the highest homology with TRPV5. <sup>(81)</sup>



**Figure 12. Mechanism of action of Klotho as a membrane and a secreted protein.** Binding of FGF23 to the membrane Klotho and FGFR coreceptor complex leads to inhibition of the synthesis of 1,25-vitamin D3 and inhibition of the expression of Na<sup>+</sup>-dependent phosphate cotransporter in the apical membrane of the proximal tubule. Secreted Klotho stimulates renal Calcium reabsorption through the TRPV5 Calcium channel.

The critical role of TRPV5 in renal Calcium handling was demonstrated by the generation of mice lacking this Calcium channel.<sup>(83)</sup> These mice displayed several phenotypic features related to a diminished active Calcium reabsorption. Additionally, a reduced trabecular and cortical bone thickness indicated disturbed bone morphology. The characteristic pore region of TRPV5 and TRPV6 is unique for its high Calcium selectivity.<sup>(84)</sup> The regulation of TRPV5 by secreted Klotho is possible thanks to its  $\beta$  glucosidase activity that participates in removal of  $\alpha$  2,6-linked sialic acid of TRPV5 in intact cells. Removal of the terminal sialic acids from N-glycans exposes underlying LacNAc for binding with galectin-1 to enhance retention of TRPV5 at the cell surface (Figure 13).

<sup>(89)</sup> The stimulatory effect of Klotho on TRPV5 is similar to its effect on TRPV6.<sup>(86)</sup>



**Figure 13. Model for the increment of TRPV5 in cell-surface abundance by Klotho.** Klotho removes terminal sialic acid from the GlcNAc  $\beta$  (1,6) branch of (tri-antennary or) tetra-antennary N-glycan of TRPV5. Removal of sialic acids exposes underlying LacNAc, a ligand for galectin-1. Binding to galectin-1 lattice at the extracellular surface leads to accumulation of functional TRPV5 on the plasma membrane.

In the absence of Klotho, TRPV 5 may not be well expressed at the luminal membrane resulting in Calcium wasting. This was demonstrated by an increased fractional excretion of calcium in Klotho-deficient mice.<sup>(81,87)</sup> The secreted form of Klotho is an important component in the calcium homeostasis in the kidney.<sup>(88)</sup>

## **2. Aim of the Study**

There are very few clinical studies that link the levels of secreted Klotho in peripheral blood and variability of phenotypes in humans, and none of them in  $\beta$ -thalassemia subjects.

This study was aimed to analyze differences in Klotho levels between cases and controls. Moreover, to find possible correlations between plasma level of Klotho protein and osteoporosis, poor muscle strength and fractures in patients with  $\beta$ -thalassemia major.

## 3. Subjects and Methods

### 3.1 Subjects

The patients with  $\beta$ -thalassemia major ( $\beta$ -TM) included in the study were attending the Thalassemia Center of the University-Hospital Sant'Anna in Ferrara (Lat 44°49' N, Long 11°37' E) or the Center of Microcytemia and Congenital Anemias, Ospedale Galliera in Genoa (Lat 44°25' N, Long 08°54' E) for comprehensive therapy. Being the Klotho protein age-sensitive, the  $\beta$ -thalassemia major patients were selected within the age-range of 30-49 years. The blood sample for biochemical assays was drawn before blood transfusion. A total of 106 patients were enrolled (62 from Ferrara and 44 from Genoa). The control group was formed by 95 healthy blood donors (52 from Ferrara and 43 from Genoa). The entire cohort was enrolled between November and February 2012. Information about major osteoporotic fractures (distal forearm, hip and vertebral) was obtained by clinical record reviews. Fractures that occurred because of a major trauma were not considered. The study protocol was approved by the Ethics Committees of the University-Hospital Sant'Anna in Ferrara and of the Ospedale Galliera in Genoa. The study complies with the World Medical Association Declaration of Helsinki, Ethical Principles for Medical Research Involving Human Subjects. An informed written consent was obtained by all individuals before enrollment.

### 3.2 Enzyme-linked Immunosorbent Assay (ELISA)

Klotho concentration in plasma was quantified using an ELISA assay (IBL Ltd, Takasaki, Japan). The assay was read at 450nm using a Victor X4 2030 Multilabel Reader (Perkin Elmer, Waltham, MA). Klotho was expressed as pg/ml. The inter-assay and intra-assay coefficients of variation were calculated (2% and 9% respectively).

### ***3.3 Dual-energy X-ray Absorptiometry (DXA) and Osteoporosis***

Dual energy X-ray absorptiometry (DXA) is a clinically proven method of measuring bone mineral density (BMD) in the lumbar spine, femur, forearm, and also whole body. <sup>(89–92)</sup>

A DXA scan uses low-energy X-rays. The machine sends X-rays from two different sources through the bone being tested. Bone blocks a certain amount of the X-rays. The more dense the bone is, the less X-rays get through to the detector. By using two different X-ray sources rather than one it greatly improves the accuracy in measuring the bone density (Figure 14).

The amount of X-rays that comes through the bone from each of the two X-ray sources is measured by a detector. This information is sent to a computer which calculates a score of the average density of the bone. A low score indicates that the bone is less dense than it should be, some material of the bone has been lost, and it is more prone to fracture.

It is used primarily in the diagnosis and management of osteoporosis and other disease states characterized by abnormal BMD, as well as to monitor response to therapy for these conditions. <sup>(93)</sup>

BMD was measured at the lumbar spine (L2-L4), femoral neck and total hip. BMD was evaluated with a Delphi QDR Series machine (Hologic Inc., Bedford, MA). The results were expressed as T-score value. The patients were divided into three categories (normal, osteopenic and osteoporotic) according to the definition of the World Health Organization: Normal when the T-score  $\geq -1.0$ , osteopenic when  $-2.5 < \text{T-score} < -1.0$  and Osteoporotic when T-score  $\leq -2.5$ . The BMD was assessed in  $\beta$ -thalassemia patients but not in controls.

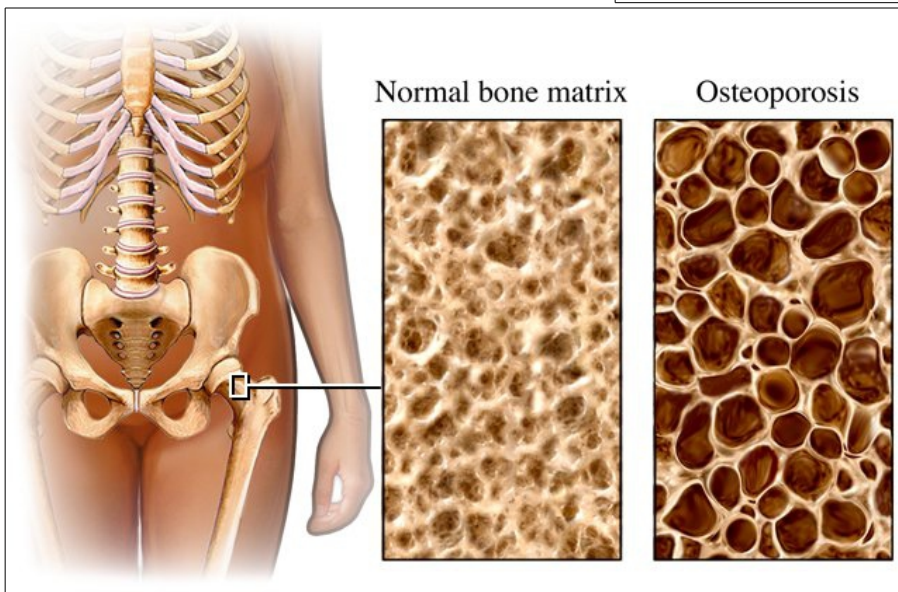
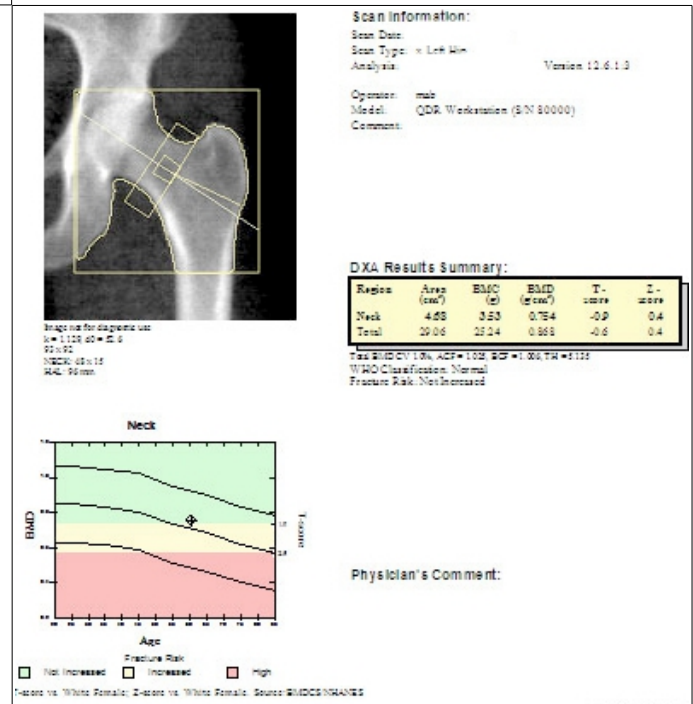


**Figure 14.**

**Above:** The DXA scan while operating.

**On the right:** The result of a DXA scan at the hip. The result provides information about the value of the bone mineral density and the T-score.

**Below:** comparison of a normal bone matrix and an osteoporotic one at the femor neck.





### ***3.4 Biomarkers***

Biochemical analysis including serum levels of intact parathyroid hormone (PTH), 25-hydroxy vitamin D (Vitamin D), serum calcium (Ca), phosphate (P), total alkaline phosphatase (ALP), ferritin, creatinine, bilirubin (total and fractionated) were measured by standard clinical techniques. The commercial kits were provided by Roche Diagnostics (Mannheim, Germany) except for Vitamin D (DiaSorin Inc., Stillwater, MI).

### ***3.5 Hand-grip Strength Test***

Hand-grip strength was measured using a JAMAR hand dynamometer (Model BK-7498, Fred Sammons Inc, Brookfield, IL) just before blood transfusion (Figure 15). The examiner explained what the test consisted in. The candidate was asked to indicate any pain at one hand or both, or any kind of injury or surgery that could affect the result of the test. The candidate was seated in front of a table with the elbow and forearm laying on it. The examiner adjusted the handle of the dynamometer such that the proximal interphalangeal joints of the hand were bent to 90 degrees. Once the preparatory step was completed, the candidate was asked to handle the dynamometer and, when ready, to squeeze it until the hand was closed, then release. Two sessions were performed with a rest period of 10 minutes. Both hands were tested. The best value was used for the analysis. To account for difference in body size, the hand-grip value was divided by Body Mass Index (BMI) calculated by dividing body weight (kg) by height squared ( $\text{cm}^2$ ). The normalized hand-grip strength was used in the analysis and indicated as hand-grip/kg.



**Figure 15. Photo of the JAMAR hand dynamometer (Model BK-7498, Fred Sammons Inc, Brookfield, IL).** Thanks to the guide and the handle clip, the handle of the dynamometer could be adjusted into five different positions such that the proximal interphalangeal joints of the hand were bent to 90 degrees.

### ***3.6 Statistical Analysis***

Differences in quantitative data were analyzed with Student's *t*-test and the values were given as mean±standard deviation (SD). The Shapiro-Wilk test was applied to check for normal distribution and the Bartlett test to check for equal variances across samples. When not normally distributed, values were logarithmically transformed. If the transformation was not possible, the non-parametric Mann–Whitney *U*-test was used. Fisher's exact test was used to examine the significance of qualitative data by the association in 2×2 contingency tables. Linear regression was performed to analyze the association of the outcome with multi-level variables. Odds Ratio (OR) was reported with a 95% interval of confidence (95% CI). Pearson's correlation coefficient (*R*) was computed for the parametric estimates of the association level between variables. Statistical analysis was performed using the software R version 2.15.2 ([www.r-project.org](http://www.r-project.org)). The piecewise regression was performed using the R package 'segmented' (version 0.2-8.4). The R package 'pwr' (version 1.1.1) was used to check the statistical power based on the sample size, standard deviation and mean to have type II error ( $\beta$ ) > 80%. A *p*-value < 0.05 (type I error or alpha < 0.05) was considered statistically significant.

## 4. Results

### 4.1 Patients

A total of 106 patients were enrolled (62 from Ferrara and 44 from Genoa). The control group was formed by 95 healthy blood donors (52 from Ferrara and 43 from Genoa). The age of the  $\beta$ -TM patients (n=106) was  $38.6\pm 6.5$  years (mean $\pm$ sd) while the control group (n=95) mean age was  $40.9\pm 7.8$  years. No statistical age difference was observed between the groups.

### 4.2 Klotho in the Peripheral Blood

The Klotho protein level was significantly lower in  $\beta$ -TM patients than in healthy controls ( $558\pm 160$  vs.  $618\pm 141$  pg/ml of protein respectively,  $p=0.0021$ ). Stratifying by sex, no significant difference was observed neither in  $\beta$ -TM patients (42 M:  $547\pm 122$  vs. 63 F:  $574\pm 185$ ,  $p=0.35$ ) nor in healthy controls (70 M:  $608\pm 132$  vs. 25 F:  $628\pm 174$ ,  $p=0.52$ ). Klotho was found to correlate directly with ALP, Ca, total bilirubin levels only in  $\beta$ -TM patients, while inversely correlated with age in both groups. (see Table 1) The significant correlation between Klotho and ALP was observed only in patients who had not received bisphosphonate (n=75,  $R=0.37$ ,  $p=0.017$ ). Moreover, adjusting for PTH and Vitamin D levels, through a linear regression model, the correlation between Klotho and ALP levels was still significant ( $\beta=0.61$ ,  $p=0.0007$ ). PTH and Vitamin D were observed to be inversely correlated ( $R=-0.41$ ,  $p<0.0001$ ). No other significant correlation among biomarkers was observed. Patients in Vitamin D supplementation did not show a difference in Klotho levels when compared to patients without supplementation ( $546\pm 162$  vs.  $612\pm 164$ ,  $p=0.062$ ). Patients in bisphosphonate therapy, compared to non in therapy, did not show any difference in Klotho levels ( $560\pm 152$  vs.  $573\pm 170$ ,  $p=0.73$ ).

**Table 1.** Characteristics of  $\beta$ -thalassemia major patients (n=106) and their correlation values with Klotho level.

	Median (25th, 75th percentile)	Klotho	p-value
		R	
BMI [Kg/m <sup>2</sup> ]	22.5 (20.4, 24.3)	-0.06	0.62
Age [years]	38 (34, 42)	-0.37	0.005
Ca [mmol/L]	2.4 (2.2, 9.1)	0.25	0.008
P [mg/dl]	3.8 (3.4, 4.4)	0.05	0.43
Vitamin D [ng/ml]	21.1 (13.8, 26.6)	0.03	0.72
PTH [pg/ml]	25.4 (20.2, 34.0)	0.06	0.52
ALP [U/L]	200.5 (142.8, 237.5)	0.39	0.002
Creatinine [mg/dl]	0.65 (0.54, 0.78)	0.01	0.93
Ferritin [ng/ml]	624.5 (415.7, 1182.0)	0.13	0.2
Bilirubin (unconjugated) [mg/dl]	0.4 (0.3, 0.5)	0.24	0.06
Bilirubin (total) [mg/dl]	1.3 (0.9, 1.9)	0.19	0.04
Hand-grip/kg <580 pg/ml	1.4 (1.1, 1.7)	0.11	0.01
Hand-grip/kg $\geq$ 580 pg/ml		-0.05	0.72

Correlation values between Klotho and continuous variables were expressed using Pearson's coefficient (R).

### ***4.3 Klotho and the Bone Status***

$\beta$ -TM patients were divided into three groups depending on the T-score value. We analyzed the relationship between Klotho and bone status at three different sites: the lumbar spine (L2-L4), femoral neck and total hip.

BMD T-score of the lumbar spine (L2-L4) showed that 9 patients were normal, 35 osteopenic and

57 osteoporotic. At the femoral neck, 16 were normal, 62 osteopenic and 23 osteoporotic. At the total hip, 13 were normal, 54 osteopenic and 34 osteoporotic.

No difference in Klotho was observed at the lumbar spine (L2-L4) and femoral neck.

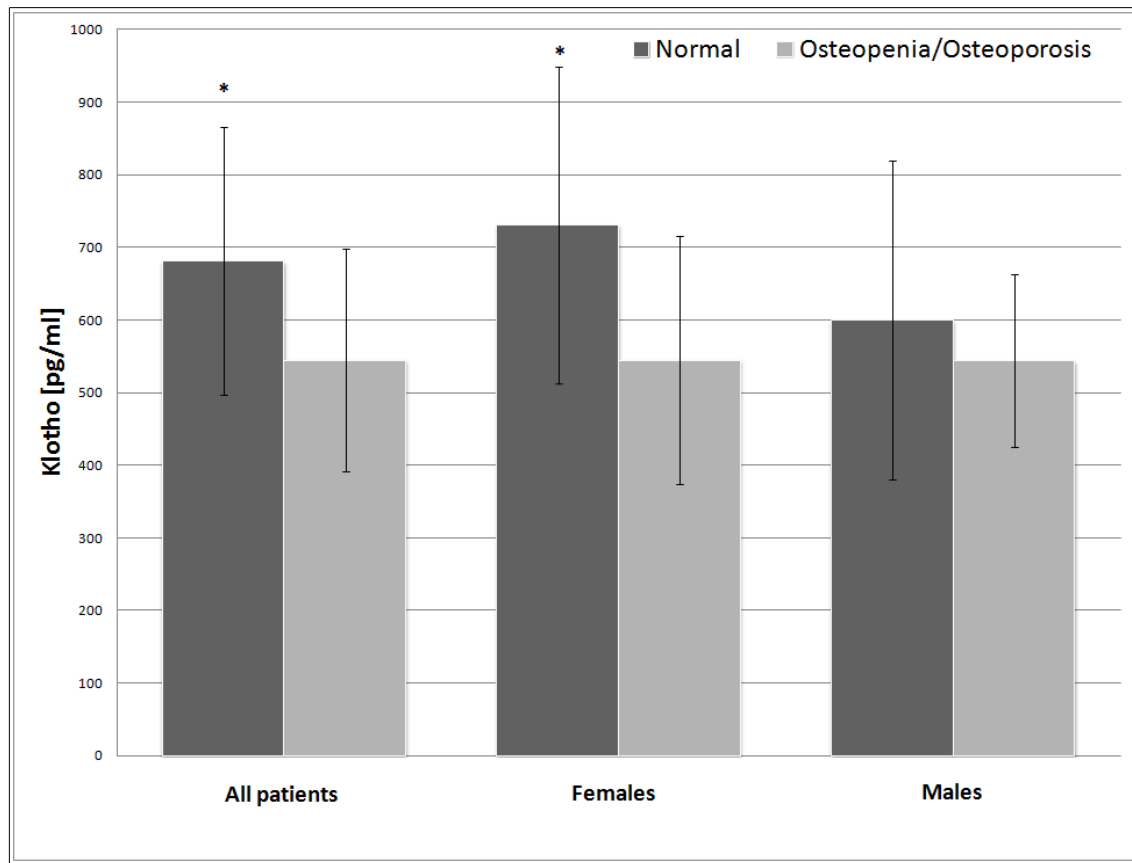
At the total hip, a significant difference in Klotho was found between normal ( $681.2 \pm 184.7$ ) osteopenic ( $549 \pm 156$ ,  $p=0.012$ ) and osteoporotic patients ( $537 \pm 149$ ,  $p=0.010$ ).

The difference between osteopenic and osteoporotic patients was not statistically significant ( $p=0.73$ ); thus, osteopenic and osteoporotic patients were grouped and analyzed as one group ( $545 \pm 153$ ,  $p=0.010$ ).

At the total hip, there was a significant difference between normal ( $n=8$ ) and osteopenic female patients ( $n=33$ ) ( $731 \pm 218$  vs  $546 \pm 177$ ,  $p=0.022$ ), and between normal and osteoporotic female patients ( $n=21$ ) ( $544 \pm 165$ ,  $p=0.031$ ). Because there was no difference between osteopenic and osteoporotic females ( $p=0.80$ ), the two groups were merged and analyzed as one group ( $545 \pm 171$ ,  $p=0.030$ ).

Conversely, at the total hip there was no difference among males (Figure 16).

Being either on Vitamin D or bisphosphonate therapy did not cause any significant difference within the normal, osteopenic and osteoporotic groups. Information about fragility fractures was obtained for 61  $\beta$ -TM patients (59.4%). Of those, 29 patients had a history of at least one fragility fracture (either of vertebrae, distal forearm or femur) at the time of enrollment. Klotho was significantly lower in patients presenting with a fragility fracture than in the 32 subjects who had not fractures ( $520 \pm 160$  vs  $597 \pm 116$  respectively,  $p=0.031$ ).  $\beta$ -TM patients with Klotho lower than 520 pg/ml had nearly a 4-fold higher probability of presenting with a fragility fracture (OR=3.84, 95% CI 1.2 – 14.2,  $p=0.012$ ).

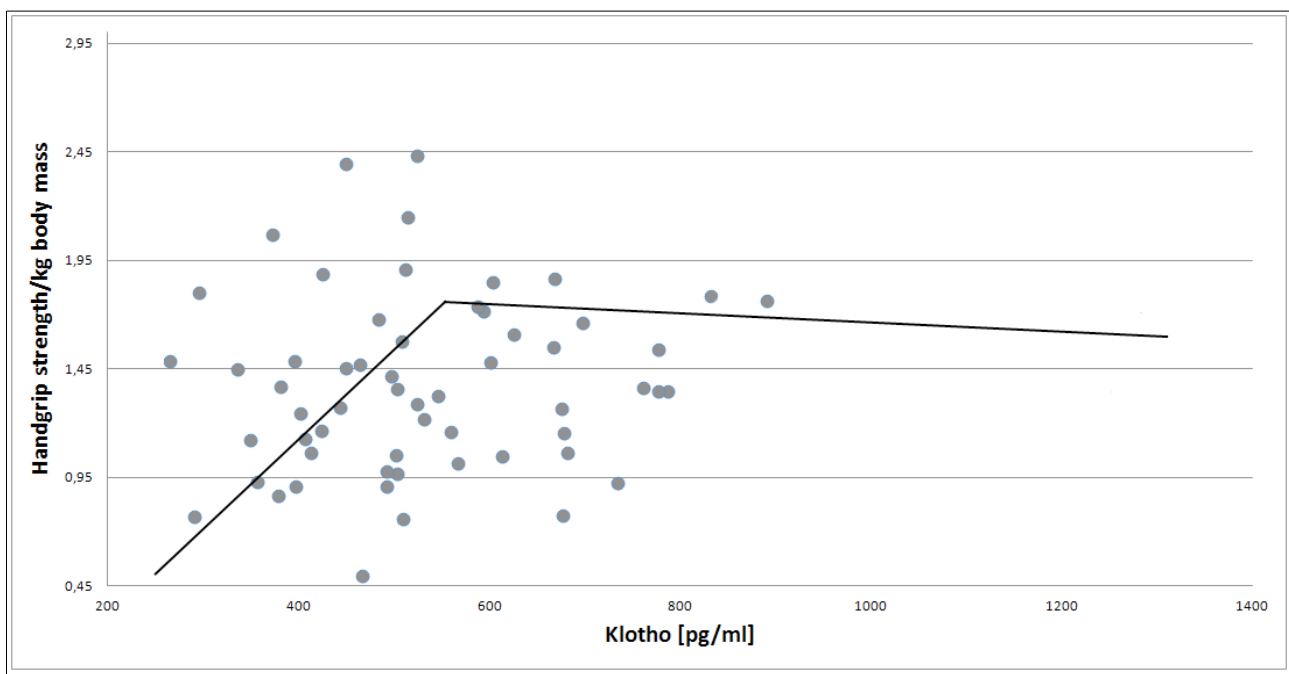


**Figure 16.** Klotho concentration in relation to the DXA T-score values at total hip of  $\beta$ -Thalassemia patients (all patients) and stratified by sex. Normal:  $\beta$ -Thalassemia patients with T-score  $\geq -1$ ; Osteopenia/Osteoporosis:  $< -1$ ; p: p-value. \*: statistical difference between normal bone and osteopenic/osteoporotic patients ( $p=0.010$ ) and between normal bone female and osteoporotic female patients ( $p=0.030$ ).

#### ***4.4 Klotho and the Hand-grip Strength***

The Hand-grip test was performed on 63  $\beta$ -TM patients. A scatter-plot analysis suggested the possibility that the relationship between Klotho and the hand-grip/kg strength was made of two different linear correlations. A piecewise regression was used to examine this possibility and estimate a break-point value that represents the change in the linear correlation slope. Up to an estimated Klotho threshold value of  $580 \pm 149$  pg/ml, a significant linear correlation was observed between Klotho and the normalized hand-grip strength ( $p=0.019$ ). For Klotho values above 580

pg/ml the linear correlation was not statistically significant. A Pearson coefficient was calculated for each segment of correlation. For the segment with a threshold value  $< 580$  pg/ml,  $R=0.11$  ( $p=0.019$ ). For the segment with a threshold value  $\geq 580$  pg/ml,  $R=-0.05$  ( $p=0.72$ ) (Figure 17). The same analysis was performed normalizing the hand-grip strength by age. The estimated age-normalized break-point was  $552\pm220$  pg/ml showing similar correlations as in the hand-grip/kg strength analysis. Moreover, male  $\beta$ -TM patients had a significantly stronger hand-grip/kg compared to female  $\beta$ -TM patients (42 M:  $1.84\pm0.56$  vs. 63 F:  $1.19\pm0.29$ ,  $p<0.001$ ). The correlation values were adjusted by sex.



**Figure 17.** Segmented scatter-plot correlation between Klotho level in peripheral blood and hand-grip strength. Klotho threshold value:  $580\pm149$  pg/ml. Threshold value  $< 580$  pg/ml,  $R=0.11$  ( $p=0.019$ ). Threshold value  $\geq 580$  pg/ml,  $R=-0.05$  ( $p=0.72$ ).

#### ***4.5 Hand-grip Strength and Bone Status***

To clarify whether the hand-grip strength was correlated to the bone status, the group of  $\beta$ -TM patients was divided into three sub-groups depending on the T-score value. Eleven patients had a normal bone status, 32 were osteopenic and 20 osteoporotic. Through a linear regression model, no

significant difference in hang-grip/kg strength was found between patients with normal bone status compared to osteopenic ones ( $1.55 \pm 0.48$  vs.  $1.49 \pm 0.42$  respectively,  $p=0.71$ ). A trend toward significance was found between patients with normal bone status and osteoporotic ones ( $1.30 \pm 0.48$ ,  $p=0.08$ ) at the total hip.

The hang-grip/kg strength showed a linear Pearson's correlation with Ca ( $R=0.49$ ,  $p=0.0002$ ), P ( $R=-0.33$ ,  $p=0.02$ ) and creatinine ( $R=0.47$ ,  $p=0.0005$ ).



## 5. Discussion

Aging patients with  $\beta$ -TM are affected by many complications that can, for the most part, be attributed to transfusional hemosiderosis or anemia. Nevertheless, they are also often affected by sarcopenia, extra osseous calcifications, increased excretion of calcium in the urine, and, with high frequency, by osteoporosis, symptoms that cannot be easily explained by the mentioned mechanisms.

Sarcopenia and osteoporosis frequently co-exist in frail older adults, and are associated with increased risk of disability, falls and fractures. Since sarcopenia and osteoporosis share similar pathogenesis and interact in producing adverse health outcomes, Binkley *et al.* have proposed to term such combination as “dysmobility syndrome”, using an approach similar to that used to define the metabolic syndrome.<sup>(94)</sup> As well as in frail older adults, also in  $\beta$ -TM patients dysmobility syndrome appears quite frequent, and presents clinical relevance being potentially associated with increased risk of fragility fracture, disability and decreased quality of life. In older adults, the pathogenesis of dysmobility syndrome is multifactorial. Literature data indicates that also in thalassemic patients the pathogenesis of sarcopenia and osteoporosis is related to several conditions and comorbidities.<sup>(21)</sup> Although the pathogenesis of sarcopenia in thalassemic patients has not been fully investigated, recent data suggested that gonadal status may play a role.<sup>(95)</sup> Moreover, it is likely that, as well as in older adults, several other factors may contribute to sarcopenia in thalassemia patients (e.g. Vitamin D deficiency).

Disruption of the Klotho gene expression in mice results in a clinical picture similar to the one described above. We measured the soluble transmembrane protein in the plasma of 106 patients and of 95 controls, and the patients' hand-grip strength. The hand-grip strength is considered to mirror the total body muscle strength, while the patients' BMD is the best tool to assess bone mineral density and risk of fractures. To our knowledge, this is the first study to measure the circulating

hormone Klotho in  $\beta$ -TM patients.

The most important result of this study was to find that Klotho levels were lower in  $\beta$ -TM patients than in controls. This is the first time that Klotho was studied in  $\beta$ -thalassemia. Moreover, that Klotho correlated with age, calcium and total alkaline phosphatase. Hand-grip strength was significantly correlated to plasma Klotho up to a threshold of 580 pg/ml. The result is similar to the one reported by the researchers of the InCHIANTI study, who enrolled a large number of healthy individuals older than 65 years living in Tuscany, Italy.<sup>(96)</sup> In our patients we also found a significant correlation between creatinine level and hand-grip strength. In fact, serum creatinine level, deriving from the metabolism of creatine, in the absence of renal problems, is a surrogate of muscle mass.<sup>(97)</sup>

The skeletal muscle growth is regulated by a signaling pathway that involves the insulin-like growth factor 1 (IGF1) and a cascade of intracellular components like Akt/protein kinase B (Akt/PKB) and forkhead transcription factors (FoxOs) that mediate muscle mass regulation.<sup>(98,99)</sup> Activation of FoxO promoter is stimulated by Klotho<sup>(60)</sup> with a consequent activation of the manganese superoxide dismutase (SOD2) and reduced oxidative stress. Humoral Klotho is also involved in regulation of nitric oxide production in the endothelium<sup>(100,101)</sup> and has been hypothesized to have a protective role against endothelial dysfunction.

Klotho was significantly lower in female patients with osteopenia and osteoporosis measured at the total hip. No difference was found when the bone mineral status was assessed at the femoral neck or at the lumbar spine. To further characterize the association between Klotho, sarcopenia and osteoporosis, we were able to investigate and compare Klotho levels in  $\beta$ -TM patients presenting with fragility fractures and in those with no history of osteoporotic fractures. This analysis was particularly relevant since sarcopenia and osteoporosis, components of the disability syndrome, are well-recognized risk factors for fragility fractures.

We found that having a plasma Klotho lower than 520 pg/ml increased the probability of fragility

fractures of nearly 4-folds. Because Klotho decreases with age, we restricted our sample to patients of a limited age range (30-49). The finding of a lower level of Klotho in patients who had previously suffered from fractures is of great interest, although in our patients the analysis is retrospective. It would be interesting to study the association of Klotho with fractures in a prospective study.

In conclusion, in  $\beta$ -TM patients the circulating Klotho is lower than healthy controls.

This analysis suggests that age- or even a disease-related decrease of Klotho may play a key role in the chain of events producing sarcopenia and osteoporosis, components of the dysmobility syndrome. Furthermore, our data identify Klotho as a potential risk factor for dysmobility syndrome and its clinically relevant complication, namely fragility fractures.

The regulatory effects of FGF23 and Klotho, on the endocrine kidney–bone–parathyroid gland axis, in  $\beta$ -TM patients and polymorphisms of Klotho gene deserve to be further investigated.

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